A VASCULAR FUNCTION-RELEVANT TRANSCRIPTIONAL MODULE OF PERIPHERAL BLOOD MONONUCLEAR CELLS THAT IS MODIFIED IN NEURODEGENERATIVE DISEASES

Undergraduate Research Thesis

Presented in Partial Fulfillment of the Requirements for graduation "with Honors Research Distinction in Neuroscience" in the undergraduate colleges of The Ohio State University

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May 2015

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ABSTRACT

Background. Besides neuron-specific factors, vascular alterations are involved in the pathogenesis of several age-dependent central nervous system (CNS) diseases. For example, the majority of cardiovascular risk factors are present in patients with Alzheimer's disease (AD). Moreover, the levels of circulating bone marrow-derived progenitor cells increase in blood with the severity of AD, suggesting an active vascular damage and repair. Using co-expression analysis of peripheral blood mononuclear cells (PBMCs), we have previously identified a gene module centered on OLR1 (coding for LOX-1, the endothelial-specific receptor for oxidized LDL), that correlates with vascular function in normal individuals.

Objective. The goal of the current study was to verify the hypothesis that this module is also altered in blood of patients with cardiovascular and neurodegenerative diseases.

Methods. To this end, we downloaded from public databases and normalized Affymetrix GeneChips data of adult human PBMCs of patients and corresponding healthy controls. Then, we compared the expression levels of the members of a previously validated co-expression OLR1-centered module (OLRM).

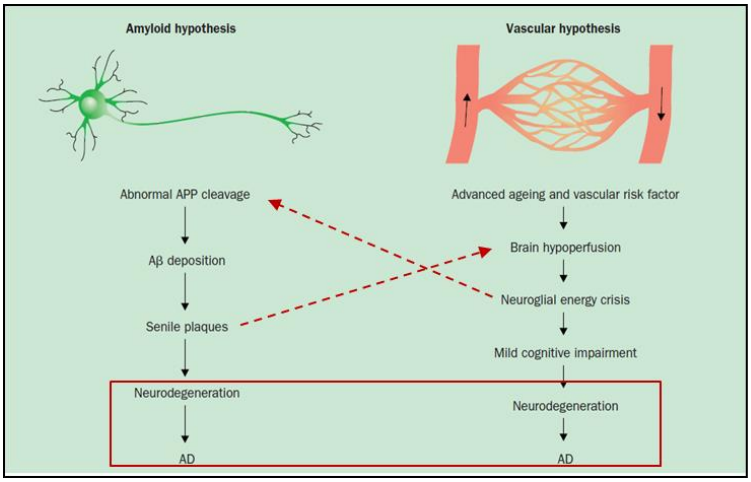
Results. The collective expression of OLRM members was significantly increased compared to controls in PBMCs from healthy pregnant women and in juvenile and adult patients with diabetes, in adult subjects with hyperlipidemia, and in seniors with AD, and was significantly decreased in women with multiple sclerosis. At the same time, changes in another gene module, centered on NOTCH4, were insignificant, confirming the specificity of the observed effects. The expression levels of OLRM members were collectively increased by statin treatment and by dietary omega-3 fatty acids containing lipids.

Conclusion. We expanded the biological validation of a peripheral blood transcriptional module that is increased in several conditions known to lead to or to derive from vascular dysfunction, including AD and MS, and to be sensitive to pharmacological (statins) and lifestyle (dietary) interventions. This suggests that OLRM qualifies as a new collective transcriptional biomarker for detection and monitoring of disease, including disorders of the CNS.

BACKGROUND AND RATIONALE

1. Neurodegenerative diseases have an essential vascular component. Neurons are extremely specialized cells, with unique energetic needs (for oxygen, glucose, etc.). Moreover, their assembling in complex multicellular structures creates additional difficulties for metabolite diffusion to and from them. For this reason, the blood vessels are intimately integrated with the neurons or neuronal bundles that are anatomically organized as 'neuro-vascular units'[1]. On the other hand, the structure and function of blood vessels, namely the vascular cells reactivity and subsequent vascular and microvascular perfusion, also essentially depend on their innervation[1]. Thus, it naturally follows that dysfunctions in one compartment translates in the malfunction of the other.

A case in point is Alzheimer's disease (AD), largely considered to be triggered by neuron-specific molecular defects, at least in the familial forms of the disease, but increasingly recognized to be triggered by neuro-inflammation[2], and/or by a concurrent vascular pathology. In fact, some investigators are convinced that AD (at least in its late-onset forms), as well as other dementias, are mainly a consequence of cerebral hypoperfusion[3] (Diagram 1).



Reported risk factors for AD

Heart-related risk factors
Congestive heart failure
Cardiac arrhythmia
Hypertension
Hypotension
Thrombotic episodes
High concentrations of homocysteine in the serum
Atrial fibrillation
Presence of *APOE* ε4 allele
Atherosclerosis

Peripheral risk factors
Smoking
Alcoholism
High serum cholesterol
High intake saturated fat
Diabetes mellitus
Haemorheological abnormalities
High cholesterol concentrations in the plasma

Diagram 1. Presumed pathological pathways leading to the development of AD according to the amyloid or vascular hypotheses, and associated risk factors. In fact, these pathways may overlap and/or cross-talk to each other, as suggested by the arrows (modified after JC de la Torre, ref.4).

Here we summarize the many apparent flaws of the 'amyloid hypothesis' of AD (following Jack C. de la Torre[4]): amyloid deposition has not yet been found to be neurotoxic in vivo; senile plaques are the *products* of sick neurons, thus they cannot also be the *cause* of their sickness, because a product is the result and not the cause of some activity; amyloid deposition in the brain does not relate with dementia severity; many patients without dementia have the same density of senile plaques as the patients with AD; amyloid deposition is not the earliest neuropathological event observed in those afflicted with the disease; alternatively, many

cognitively healthy elderly people have abundant senile plaques in the brain without signs of AD; amyloid deposition in the brain does not correlate to neuronal, metabolic, or synaptic loss; amyloid plaques can be found in other dementias, including vascular dementia. Also, in the pre-clinical research realm, experiments with transgenic mice that produce amyloid beta deposits in the brain show, as in humans, no relation between such deposits and neuronal, metabolic, or synaptic loss; the same transgenic mice also show cognitive loss before amyloid is deposited in the brain; memory loss is independent of amyloid beta overexpression in transgenic mice; transgenic mice overexpressing an isoform of human amyloid peptide show low glucose metabolism and loss of glia in the absence of amyloid deposits, which suggests a neuronal-energy deficit precedes amyloid deposition; in these mice with memory-impairment, vaccinations against amyloid over 8 weeks did not improve cognition.

2. OLR1 is a genetic risk factor for AD. An abundance of genetic, epidemiological, comorbidity, neuroimaging, and risk factors overlap (summarized in Diagram 1, left side) and concur to suggest that the primary insult in AD (in the late-onset forms at least) might be targeting the brain (micro)vasculature[4]. Among those arguments is the fact that two major genetic polymorphisms associated with the emergence of this disease are related to lipoproteins in the circulation. One is the variant ε4 of ApoE, which is a lipoprotein particle-associated protein that interacts with low-density lipoprotein (LDL) receptors on cellular surfaces, although its function is considered to be mainly related to neuro-inflammation[5]. Another gene polymorphism, and possibly related to the first[6], is one for OLR1, coding for the lectin-like oxidized low density lipoprotein receptor (LOX-1), the proposed *endothelial* 'scavenger receptor'[7].

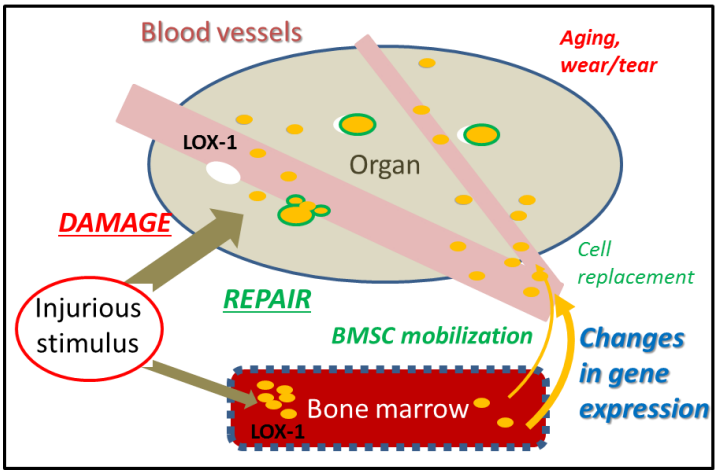


Diagram 2. Putative role of OLR1/LOX-1 in bone marrow derived stem/progenitor cells (BMSC), as mediators of damage-repair coupling in blood-perfused organs. Note that mobilization of BMSC changes the pattern of gene expression in circulating peripheral blood mononuclear cells.

3. Role of OLR1/LOX-1 in endothelial and stem cells biology. This receptor binds many other cell-damaging ligands[8], such as glycosylated LDL, as well as other advanced glycosylation end-products (AGEs) occurring in diabetes. It is stimulated by angiotensin II[9] that is excessively produced during hypertension. LOX1 mediates, via reactive oxygen species (ROS)[10], both cellular damage (at high oxLDL concentrations) and tissue repair, mainly by stimulating neovascularization (when oxLDL is in lower amounts)[11]. LOXIN is a 'splice variant'

of OLR1-derived mRNA, coding for an isoform which can heterodimerized with LOX1 to block its deleterious effects at higher oxLDL concentrations[12]. However, the actual mechanism through which LOX-1 or LOXIN influences the course of AD remains to be determined.

LOX-1 is also expressed in bone marrow-derived progenitor cells, being involved in these cells' proliferation[13] and in their recruitment to sites of injury[14]. Alternatively, LOX-1 is responsive to plasma lipid peroxidation status in a concentration-dependent manner, being detrimental for endothelial progenitor cells at higher oxLDL concentrations[15].

4. Blood contains cells with primitive characters which provide vascular protection and tissue repair. In the last decade it became clear that beside its traditional functions (e.g. carrying of nutrients and metabolic waste, protection against infections and blood loss, maintenance of its own fluidity in an optimal range, etc.), the blood has one more essential function: to contribute stem/progenitor cells from bone marrow-located reservoirs that help replace those cells lost due to wear-and-tear or to aging in various organs, including the brain (Diagram 2). This tissue-maintenance function could be acutely amplified in response to injury by the release of even more cells with a tissue-reparative function in the circulation after different forms of damage. However, due to their rarity, primitivity (i.e. un-differentiated state), and phenotypic plasticity, the characterization of these cells proved to be very difficult[16].

To address this limitation, the Moldovan lab is developing a new approach to detect and study the presence of these cells with a tissue-reparative function in peripheral blood in both humans and mice, without the need to physically isolate them, or even to know a-priori their characteristics. The method is based on the detection of their transcriptional signature in whole peripheral blood mononuclear cell (PBMC) preparations[17]. This approach is

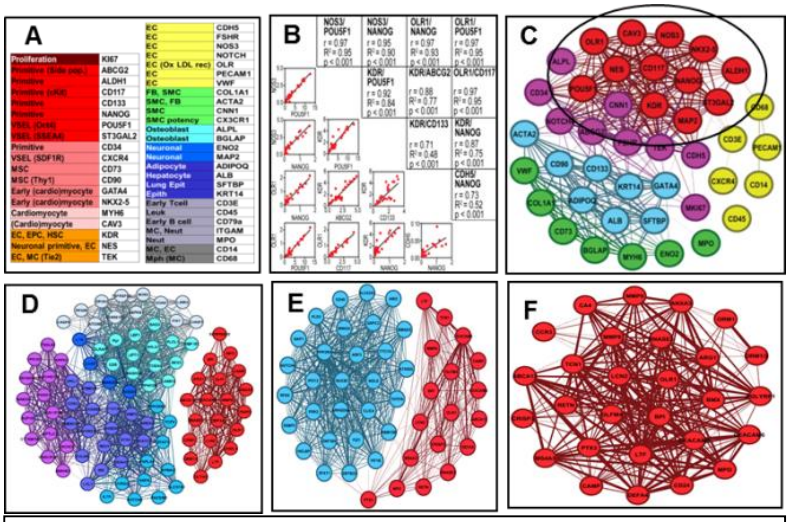


Figure 1. A module of PBMCs transcriptional network with cardiovascular function defined by co-variation analysis. A. Panel of primitive (left column) and differentiation (right column) tested genes. **B**. Example of co-variation of primitive and cardiovascular genes in PBMCs from healthy human subjects (n=27). **C.** Co-variation expression network of genes in PBMCs from normal subjects. A module composed of 15 genes (encircled) were inversely related to blood donor's age, vascular stiffness and central blood pressure, suggesting a vascular protective role**. D-F.** Expansion of this network with the 'neighborhoods' of several 'seed genes' detected by genechips in PBMC from normal blood: (D) children, (E) healthy adults, and (F) burn victims (A-C from ref. 10; D-E were obtained with the contribution of T. Kantor, ASC Honors Thesis 2014).

particularly well suited for the stem/progenitor cells system, because their state of preparedness for *future* differentiation is more inscribed in their mRNA, rather than in their proteins repertoire.

So far, our group has found a cluster of genes which in the normal human population decreases with the age of blood donors, and is inversely related to their blood pressure and vascular stiffness (indicative of a vascular-protective function), while being directly proportional to body mass index (BMI)[17].

Of note, in a retrospective cohort study relating BMI to risk of dementia in two million people from UK, it was recently found that compared with people of a healthy weight, underweight people had a 34% higher risk of dementia. Furthermore, the incidence of dementia continued to fall for every increasing BMI category, with very obese people having a 29% lower dementia risk than people of a healthy weight. These patterns persisted throughout two decades of follow-up, after adjustment for potential confounders and allowance for the J-shape association of BMI with mortality[18]. These findings are in line with other observations leading to the so-called 'obesity paradox', indicating a rather protective effect of the adipose tissue in a variety of pathologies. One explanation could be that its presence mobilizes increased levels of circulating stem/progenitor cells with reparative function, from which the organism may indirectly benefit in conditions of tissue injury.

This cluster of expressed genes (or 'metagene'[19]), is organized as a module of the PBMCs transcriptional network. In other words, among the members of the module there is a special relationship making them somehow 'equivalent' to each other. One of the most parsimonious explanations, also known as the 'principle of guilt-by-association', is that these genes contribute to a common function[20]. If so, finding new members of a given module is a way to learn more about the collective roles those genes have in the organism, or even to assign new functions to genes with known or unknown roles.

5. Expansion of the OLR1-containg gene cluster on microarrays. For this reason, our lab is performing a set of analyses aiming to detect new members of the gene module with cardiovascular function, using published studies performed on PBMC samples from normal subjects, and from patients with various cardiovascular and neurodegenerative diseases. All these studies whose rough data was uploaded in the GEO (Gene Expression Omnibus) database were performed on Affymetrix gene chips, rather than by Polymerase Chain Reaction (PCR), as in our original study[17]. The main difference is that, although more comprehensive, and thus more objective, in covering the human transcriptome, these gene 'chips' are far less sensitive than PCR at detecting low abundance RNA transcripts, as expected from those derived from rare stem/progenitor cells in the circulation. In fact, from the 15 members of our cardiovascular module, only two (OLR1 and Notch4) are detectable in PBMC from adult blood, and only four in children's blood.

Taking these genes as 'seeds' and constructing their associated co-variation networks, our group has found that while Notch4's neighborhood varies according to the source of the blood, the network of OLR1 is similar in these conditions: in normal blood from children and normal

adults, in that of patients with severe burn injury, where it is markedly increased[21], and in women with preeclampsia (a condition characterized by hypertension, vascular stiffness, increased lipoprotein oxidation and reduced placental angiogenesis)[22], where this module represent the most down-regulated genes. These last two situations can be considered as a biological 'validation' of an extended OLR1 module (OLRM), which we further used in the current study. Altogether, we formulated a gene expression co-variation module containing 29 members (Table 1), which show a relatively homogenous connectivity (Fig. 1F).

Genes with Large Fold Change				Burn Study			Preeclampsia Study			
Entrez	Affymetrix ID	Gene Symbol	Function	1-10 Day (Early) Fold Change (Compared to Controls)	11-49 Day (Late) Fold Change (Compared to Controls)	11-49 Day Fold Change (Compared to Early)	Early onset Fold Change (Compared to Controls)	p-value	Late onset Fold Change (Compared to Controls)	p-value
4317	207329_at	MMP8	Plays an important role in plaque angiogenesis	**2.63**	**3.32**	0.68	-	-	1.99	0.037
4057	202018_s_at	LTF	Highly expressed in non-healing wound exudates	**2.36**	**3.09**	0.73	-	-	2.03	0.001
3934	212531_at	LCN2	An independent renal predictor of incidence of AKI after surgical abdominal aortic aneurysm (AAA) repair	**2.30**	**3.20**	0.89	1.58	0.098	1.85	0.001
10562	212768_s_at	OLFM4	Marker of intestinal stem cells	**1.96**	**2.39**	0.43	-	-	1.88	0.077
10321	207802_at	CRISP3	Specific neutrophil granules protein, secreted as an extracellular matrix component	**1.51**	**1.12**	-0.39	-	-	1.83	0.052
671	205557_at	BPI	Antibacterial activity against Gram-negative bacteria	**1.43**	**2.43**	**1.00**	2	0.021	2.01	0.005
1669	207269_at	DEFA4	Neutrophil protein with antimicrobial activity against Gram-negative bacteria	0.55	**2.66**	**2.11**	2.36	0.011	2.49	0.001
4680	211657_at	CEACAM6	Implicated in cell adhesion, cellular invasiveness, angiogenesis, and inflammation	0.45	**2.42**	**1.96**	2.1	0.041	1.99	0.023
6037	206851_at	RNASE3	Eosinophil major basic protein with pro-angiogenic effects	0.54	**1.42**	0.89	-	-	1.89	0.027
154664	1553605_a_at	ABCA13	Markers of hMSC	0.18	**1.16**	0.98	1.56	0.086	-	-
820	210244_at	CAMP	Induces angiogenesis via PGE2-EP3 signaling in endothelial cells	0.59	**1.06**	0.47	-	-	1.66	0.002
4318	203936_s_at	MMP9	Involved in mobilization of hematopoietic progenitor cells from bone marrow as well as embryonic development, reproduction, and tissue remodeling through ECM degradation	**2.56**	**2.56**	-0.01	1.62	0.039	-	-
8993	207384_at	PGLYRP1	Binds to peptidoglycan of bacteria involved in human atherosclerotic lesions. Increasing levels associated with coronary artery calcium and aortic wall thickness	**1.69**	**2.12**	0.43	1.59	0.007	-	-
762	206209_s_at	CA4	Encodes an isozyme expressed on luminal surfaces of pulmonary and other capillaries and proximal renal tubules. Related to Ocular Hypotension	**1.54**	**1.37**	-0.17	1.52	0.040	-	-
660	206464_at	BMX	Plays a critical role in TNF-induced angiogenesis, and implicated in the signaling of TEK and FLT1 receptors, 2 important receptor families essential for angiogenesis	**1.54**	**1.61**	0.07	1.5	0.033	-	-
56729	220570_at	RETN	Resistin levels correlate with oxidative stress and myocardial injury in cardiac surgery patients. It may serve as a useful biomarker for ischemia-reperfusion injury	**1.53**	**1.63**	0.09	-	-	1.53	0.060
6947	205513_at	TCN1	Influences human vitamin B-12 levels which have shown to be implicated in congenital heart disease via the folate metabolism pathway	**1.47**	**2.29**	0.82	1.75	0.012	1.68	0.008
1088	206676_at	CEACAM8	Leukocyte activation marker. Increased leukocyte activation is correlated with coronary artery disease, plaque destabilization, and vascular cell dysfunction	**1.18**	**2.86**	**1.68**	1.9	0.051	2.09	0.004
1232	208304_at	CCR3	CCR3-dependent chemokine interactions regulate endogenous migration of CD34+ progenitors from bone marrow to ischemic but not to normal myocardium	-1.01	-0.90	0.10	1.84	0.000	1.64	0.001
260429	1552348_at	PRSS33	Serine, protease predominantly expressed in macrophages	-1.02	-0.47	0.55	-	-	1.73	0.087
100133941	216379_x_at	CD24	Interacts with P-selectin which mediates rapid rolling of leukocytes over vascular surfaces during the initial steps in inflammation	0.70	**1.90**	**1.19**	1.63	0.039	1.52	0.033
932	210254_at	MS4A3	Hematopoietic stem cell cycle regulator	-0.05	**1.79**	**1.84**	1.83	0.096	1.63	0.085
5806	206157_at	PTX3	Modulates inflammatory processes, angiogenesis, atherosclerotic lesion development, and ECM formation. Released by vascular wall cells as an inflammatory marker	0.44	**1.18**	0.74	1.57	0.045	1.69	0.002
383	206177_s_at	ARG1	Reduces nitric oxide production and impairs endothelial function	**2.68**	**2.74**	0.05	-	-	-	-
306	209369_at	ANXA3	Potential angiogenic mediator	**2.21**	**2.31**	0.10	-	-	-	-
5004/5005	205041_s_at	ORM1/2	Acute phase reactant and bimodal regulator of angiogenesis	**1.00**	**1.06**	0.06	-	-	-	-
4353	203949_at	MPO	Leukocyte activation marker. Increased leukocyte activation is correlated with coronary artery disease, plaque destabilization, and vascular cell dysfunction	0.39	**1.61**	**1.22**	-	-	-	-
5004	205040_at	ORM1	Acute phase reactant and bimodal regulator of angiogenesis	0.98	**1.16**	0.18	-	-	-	-
1116	209395_at	CHI3L1	Expressed by macrophages, chondrocytes, and vascular SMC; it is a potent angiogenic factor	-0.97	0.03	**1.00**	-	-	-	-

Table 1. Genes composing the OLR1/LOX-1 module in PBMCs.

6. Goal of current study. In line with the previous effort to expand the PCR-based cardiovascular module, the goal of this project was to further validate the OLRM against other cardiovascular conditions, and to assess its involvement in neuro-degenerative diseases.

METHODS

Studies Used: From the National Center for Biotechnology Information's Gene Expression Omnibus (GEO) public database, we assembled a collection of Affymetrix GeneChip® Human Genome U133 Plus 2.0 arrays (platform GPL570). The following studies were used: GSE17449, GSE9006, GSE15932, GSE55100, GSE11393, GSE29680, GSE22255, GSE48060, GSE27034, GSE66134, and GSE18309. Information regarding these studies can be found in Table 2 on the next page.

Figure #	Title	GSE #	Link
2	OLRM in pregnant women and impact of multiple sclerosis (MS).	17449	http://www.ncbi.nlm.nih.gov/pubmed/20126412
3a	Amplification of OLRM expression in children with TIID	9006	http://www.ncbi.nlm.nih.gov/pubmed/17595242
3b	Less coordinated increase in the expression OLR cluster members in adults with TIIB.	15932	http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE15932
4a	Amplified OLRM expression in adult Type I diabetic patients.	55100	http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE55100
4b	Minimal changes in the expression levels of genes of a NOTCH4-centered transcriptional cluster in Type I diabetes	55100	http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE55100
5a	OLRM expression in human subjects with hyperlipidemia	11393	http://www.ncbi.nlm.nih.gov/pubmed/18681780
5b	OLRM expression in human subjects with Alzheimers receiving omega-3 treatment	29680	http://www.ncbi.nlm.nih.gov/pubmed/22545106
6a	Reflection of ischemic stroke on OLRM expression levels in PBMCs	22255	http://www.ncbi.nlm.nih.gov/pubmed/22453632
6b	Reflection of acute myocardial infarction on OLRM expression levels in PBMCs	48060	http://www.ncbi.nlm.nih.gov/pubmed/24801707
6c	Reflection of peripheral artery disease on OLRM expression levels in PBMCs	27034	http://www.ncbi.nlm.nih.gov/pubmed/22409835
7	Expression of OLRM genes in age-dependent cognitive disorders	66134	http://www.ncbi.nlm.nih.gov/pubmed/17215369
8	Whole Blood controls for the OLRM modifications in AD	18309	http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE18309

Table 2. Database sources used for preparation of the figures.

Presence Score Detection: In order to determine presence scores (detection levels), Affymetrix Expression Console was used to perform MAS5 normalization. Certain genes were represented by more than one probe set, in which case the probe set with the highest presence score and signal (expression value) was used, while the rest were discarded. 'Presence Score' was calculated by percentage of all arrays in a study. If scores were 'Marginal', they were considered 'Absent' and discarded.

SCAN Normalization: The Single-Channel-Array-Normalization (SCAN) method was used to normalize microarrays. This eliminated batch effects. Instead of the group of chips being analyzed, this method normalizes each chip to itself. This enabled the addition of microarrays to any individual study without affecting the normalization procedure. This method produced intensity values which were then used for co-variation analysis.

Matrix and Network Construction: Partek Discovery Suite was used to perform Pearson correlation and Matrix analysis. Matlab Software and Ohio Supercomputer Services (OSC) were used to perform Clique Mining analysis. In order to find patterns in the gene co-expression matrix data we generated, we utilized the network mining and merging workflow described by Xiang et al.[23] We created an unweighted graph from a gene co-expression data set by generating an edge between any two genes with an absolute correlation value greater than 0.6. We then applied the Bron-Kerbosh algorithm[24] to generate all maximal cliques. The network merge approach[23] was applied to these cliques, using various density thresholds depending on the study. This guaranteed each resulting sub-network induced a sub-matrix with an average correlation value greater than the threshold used on the original gene co-expression matrix. Gephi was finally used to visualize the discovered networks.

Data Analysis: T-tests, linear regressions, and standard deviations were calculated using Microsoft Excel 2010. $p < 0.05$ was considered significant in all of the statistical analyses.

RESULTS

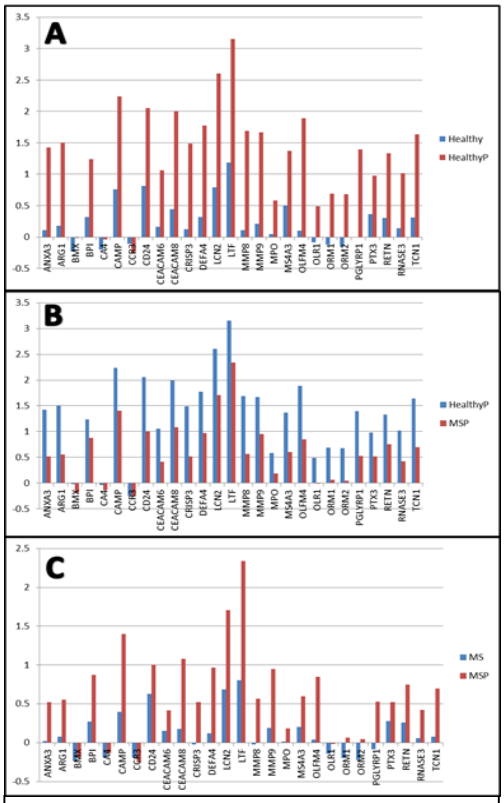


Figure 2. OLRM in pregnant women and impact of multiple sclerosis (MS). A. Pregnancy dramatically increases the expression of all OLRM members**. B.** Mothers with MS present a decrease in OLRM expression as compared to normal pregnancy**. C.** However, pregnant MS women have substantially higher expression in OLRM as compared to non-pregnant MS female patients.

1. OLRM in development-related pathology.

1.a Pregnancy and multiple sclerosis (MS).

Pregnancy is associated with higher circulating levels of progenitor cells, deemed to be mostly of fetal –rather than maternal– origin[25]. These circulating progenitors seem to play a protective/reparative role for the mother's tissues, such as the heart[26] and brain[27]. Moreover, pregnancy was shown to ease the course of some severe diseases, such as multiple sclerosis (MS)[28]. This makes it likely that the same progenitor cell pool in the mother's blood could help repair the damage of an ongoing pathological condition. However, these circulating fetal cells alone expectedly might not eliminate a maternal disease, particularly if it is related to the pregnancy itself, but still ease its course.

Recent studies suggest that the placenta of pregnant women with PE express high levels of lectin-like oxidized LDL receptor-1 (LOX-1), which induces endothelial dysfunction by increasing reactive oxygen species (ROS) and decreasing intracellular NO[29].

The observation that the OLRM is decreased in preeclampsia[30], prompted us to verify whether the amplitude of this gene cluster is increased in women with normal pregnancy in the first place. We found this to indeed be the case, as shown in Fig. 2A, which illustrates the dramatic increase of the module in the PBMCs from pregnant women, as compared with an age-matched control group. The very good linearity (regression R^2=0.98, not shown) indicates that all genes *coincidently* are (and thus non-randomly) modified and proportionally changed in the same direction, as expected from a tightly coupled transcriptional module.

Next, we compared the OLRM in women with normal pregnancies and those pregnant women suffering from MS. This time, all members of the OLRM presented smaller relative levels of expression, but again with a very good proportionality in the changes (Fig. 2B), indicative of depletion in the peripheral blood of these women of the OLRM-generating cells, or altering of their function and/or transcriptional profile. However, when we compared pregnant women with MS with non-pregnant MS patients (Fig. 2C), those bearing a child presented much higher (and again proportionally-changed) values of the OLRM expression. This is in agreement

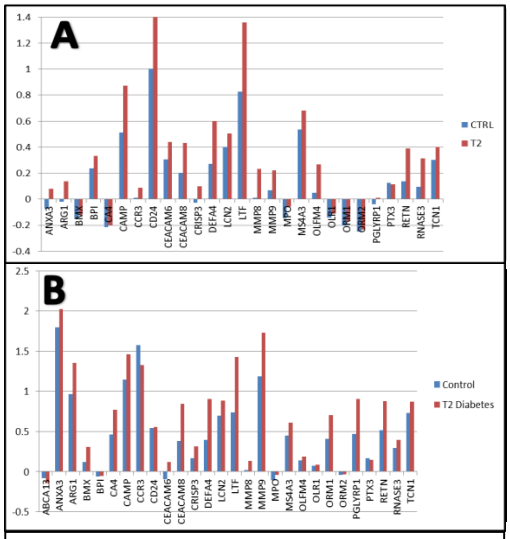


Figure 3. Effect of diabetes on OLRM. A. Amplification of OLRM expression in children with TIID. **B.** Less coordinated increase in the expression OLR cluster members in adults with TIIB.

with a model in which the pregnancy itself provides protection and/or repairing activity in women with MS, presumably helping with the easing of their symptoms.

1.b. OLRM in children's blood. Previously we found that, although quite insensitive to detecting the expression of low abundance transcripts, the microarrays showed more members of the cardiovascular module to be present in children's blood than in adults (Fig 1D). This is consistent with the idea that, corresponding to this developmental stage, juvenile blood contains more primitive and/or less differentiated cells.

Next, we analyzed the OLRM membership in blood of children with acquired (Type II) diabetes. This condition is known to lead to a decrease in circulating progenitor cells[31], however when considering more subtle diabetes-related side effects, such as diabetic retinopathy, these cells were often found to be increased[32-34]. In our study, all members of the OLRM were larger in diabetic children (Fig. 3A), although the amplitude of this effect might have been modified (or, likely reduced) by the administered insulin and/or by other anti-diabetic medication.

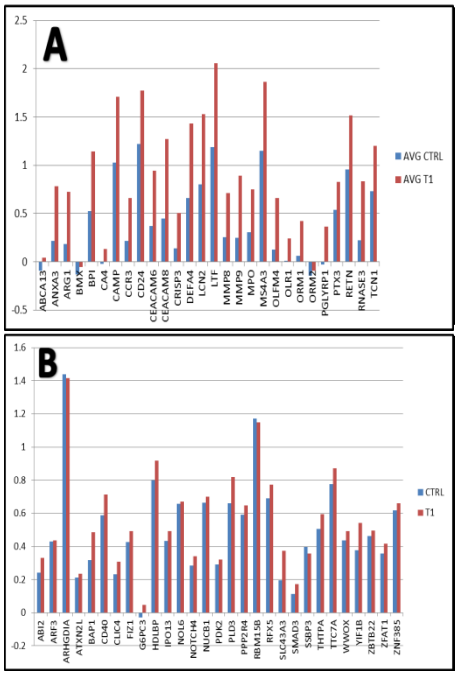


Figure 4. Specificity of OLRM modifications in diabetes. A. Amplified OLRM expression in adult Type I diabetic patients. **B.** Minimal changes in the expression levels of genes of a NOTCH4-centered transcriptional cluster in blood samples from the same patient population.

2. OLRM in adult vascular heath and disease.

2.a. Diabetes. As a major risk factor for cardiovascular diseases, diabetes' impact on the circulating cell system with vascular-protective role, and its associated transcriptional profile, deserves a special consideration. For this reason, we downloaded more databases from studies comparing the effect of both types of diabetes on gene expression in PBMCs. The changes in the OLRM observed in Type II diabetic children were clearly present in adults as well (Fig. 3B). However, the proportionality among individual genes was less pronounced than in the previous studies, possibly due to either the result of medication or of the concurrent pathologies these adult patients might have.

When we compared the impact of Type I diabetes on the expression of the OLRM metagene in adult human patients, the results were even more impressive, both in amplitude (Fig. 4A) and in linearity of co-variation (not shown). To verify the specificity of

this effect, we performed exactly the same analysis on the genes that belong to the Notch4 module (Fig.1E), and we found that the modifications in this gene cluster in adults with Type I diabetes were minimal (Fig. 4B).

2.b. Hyperlipidemia. The other major co-morbidity in the development of cardiovascular diseases is hyperlipidemia, either with increased low-density lipoprotein (LDL) cholesterol levels, or with increased triglycerides (associated with the very large low density lipoproteins, or VLDL). Assessing the influence of lipid disturbances on the OLRM structure has twofold value. First, it goes in line with the previous thinking, namely that these metabolic anomalies that alter the frequency in the peripheral blood[35] and/or functions[36] of the circulating progenitor cells system should be reflected in the express genes. Second, we may learn more about the very reason of co-variation in the expression of different genes with OLR1, the gene coding for the *oxidized LDL*. However, the latter assumption depends on whether increased LDL particles in plasma also mean an augmentation of their lipid peroxidation, which might or might not be the case, given the involvement of endogenous and exogenous (e.g. dietary) antioxidants.

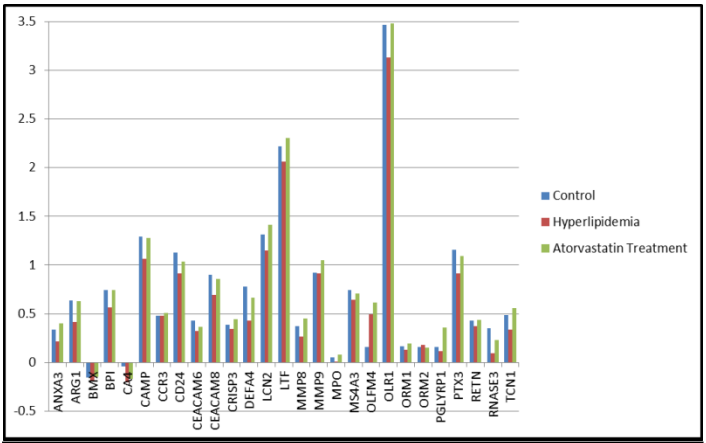


Figure 5. OLRM expression in human subjects with hyperlipidemia, and effect of statin treatment. OLRM expression before and after statin treatment in a human population with familial hypercholesterolemia, as compared with normo-lipidemic controls.

We analyzed the OLRM in a population with familial hypercholesterolemia, before and after institution of an efficient treatment with atorvastatin, one of the most frequently prescribed statins. Compared with age-matched controls, the hyperlipidemics had slightly but uniformly decreased values of OLRM members (Fig. 5A). After treatment, these values increased to levels making the differences completely disappear (Fig. 5A), as an indication of both specificity and sensitivity of the effect.

Here should be underlined the power of our covariation analysis, as compared to single-molecular markers: while the absolute changes of gene members of OLRM could be indeed low and/or *individually non-significant*, they have a strong statistical significance *as a group* (not shown).

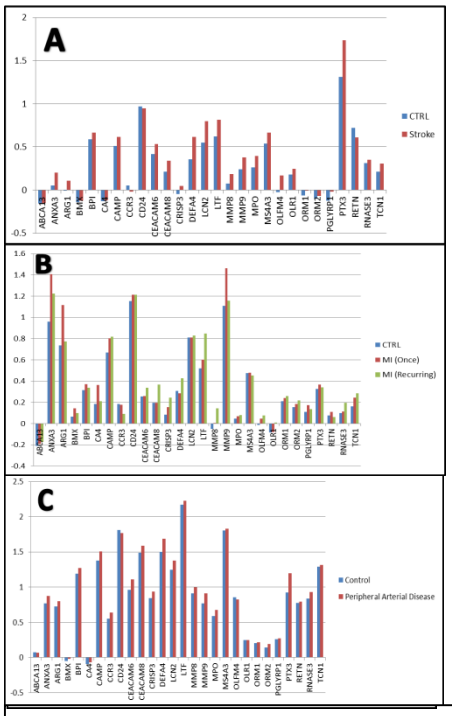
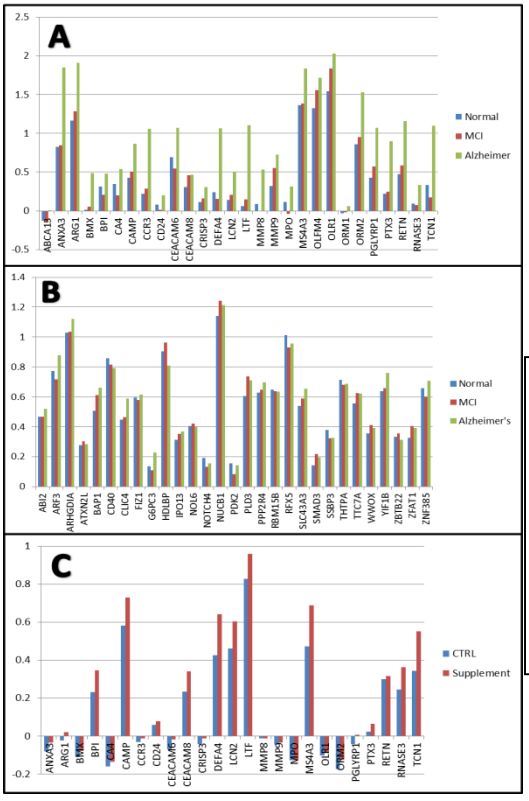


Figure 6. Reflection of large-vessel cardiovascular pathology on OLRM expression levels in PBMCs. A. Comparison of OLRM in patients with and without ischemic stroke**. B.** Changes of OLRM with AMI (one-time or recurrent). **C.** Expression of OLRM module members in patients with peripheral artery disease as compared with a normal control population.

2.c. OLRM in cerebro- and cardio-vascular pathology. Next, we asked the question as to whether the systemic changes in proportions of cells with primitive characters (interpreted as mobilization of bone marrow derived progenitor cells) occurring in peripheral blood after major acute vascular events such as stroke[37] or myocardial infarction[38] are reflected in corresponding modifications in the OLRM.

To this end, we compared OLRM-related gene expression profiling in patients with acute ischemic stroke (Fig. 6A), PBMCs samples from one-time and recurrent myocardial infarction patients, and from peripheral arterial disease patients with the corresponding matched controls. Overall, the differences were smaller and less coordinated than in some of the previous comparisons, but still statistically significant (better seen in stroke and one-time infarction) when compared as groups, rather than individually. Of note, all these blood samples were collected from patients pharmacologically treated for their condition (often with either disease specific treatment such as insulin for diabetes[39] or with poly-medication), interventions which are known to affect the circulating progenitor cells, thus possibly explaining the small amplitude of the observed effects.



In acute myocardial infarction (AMI), the changes in the OLRM were more notable in those patients with the first attack, rather those with recurrent presentations (Fig. 6B). Also, given the known increase in *soluble* LOX-1 (sLOX-1) in peripheral artery disease associated with diabetes [40], we sought to determine the changes, if any, in OLRM expression in PBMCs. The results show that a change does happen,

Figure 7. Expression of OLRM genes in age-dependent cognitive disorders. A. Comparison of OLRM changes in PBMCs with mild cognitive impairment (MCI) of blood donors, and with Alzheimer's Disease (AD). This graded modification indicates that OLRM reflects a disease stage-dependent process. **B.** Comparison for the Notch4 cluster members in the same patient population. **C**. Beneficial effect of an omega-3 enriched diet on OLRM in PBMCs of AD patients.

although at a very small level (Fig. 6C). Supposedly, the observed association with sLOX-1 might derive from the impact of diabetes itself, rather than the vascular dysfunction.

3. OLRM in age- and vascular-dependent neuro-degenerative diseases.

3.a. Alzheimer's disease (AD). As detailed in the background, the pathogenesis of AD has a substantial cerebrovascular component. If a vascular damage/repair cycle is ongoing, this could be translated into a modified profile of relevant genes in the blood cells. To test this hypothesis, we analyzed gene expression data from patients with mild cognitive impairment (MCI), AD and Parkinson's disease (PD). Fig. 7A shows a progressive, severity dependent increase in overall expression of OLRM genes (although MCI did not reach the statistical significance). Remarkably, this change was specific for OLRM, because the Notch4 module again did not show comparable changes in these patient populations (Fig. 7B). Interestingly, a diet rich in omega-3 poly-unsaturated fat tended to further increase OLRM as compared to untreated subjects.

When detection of the same genes was attempted in whole blood, rather than in PBMCs, the differences between AD and controls became minimal (Fig. 8A), arguing for a 'dilution' of the target translated genes in the abundant erythrocyte and neutrophil-derived mRNAs.

Finally, much less substantial –if any– differences were found between OLRM genes in PBMCs from PD and normal age-matched controls (Fig. 8B), in support for the specificity of this metagene for a vascular, rather neuronal, pathology.

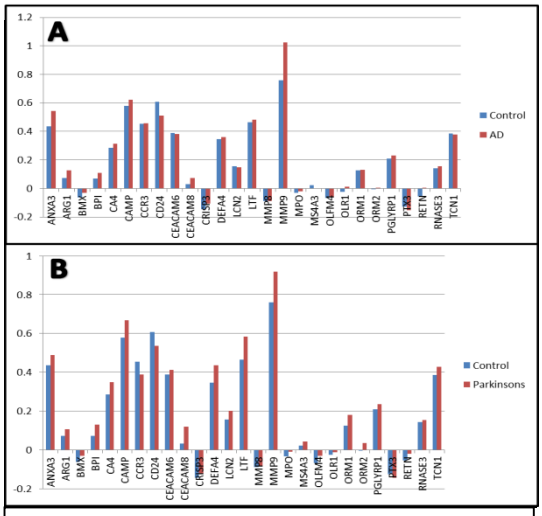


Figure 8. Further controls for the OLRM modifications in AD. A. No changes were seen when whole blood from AD patients was compared with normal controls, suggesting an mRNA dilution effect. **B.** Minimal increase in OLRM in PBMCs from Parkinson's disease patients.

DISCUSSION

In this section we'll comment on the main results of the study, and on their relevance for the field. First, we found that the OLRM indeed behaves collectively, as expected from a tightly-coupled 'metagene', displaying strong covariation in comparisons leading to either very large (e.g. pregnancy, diabetes, AD), or small changes (acute vascular events, hyperlipidemia, dietary interventions).

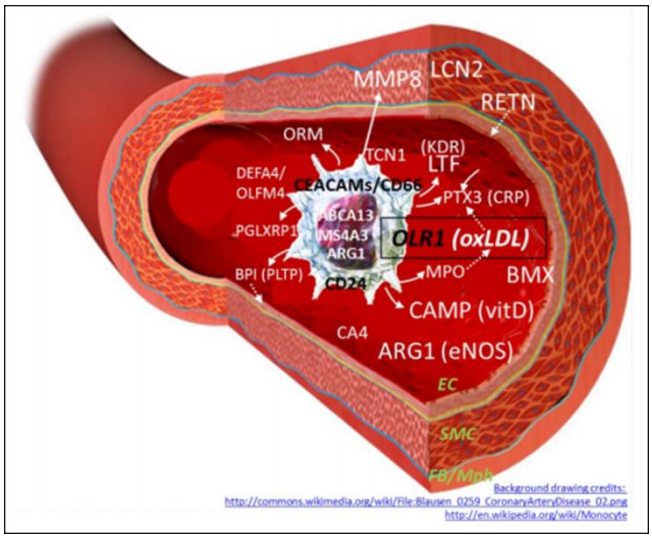


Diagram 3. Cardiovascular roles of OLRM members. The functions of these genes are given in Table 1. Projection of their functions over the vascular wall structure, as seen interacting with a generic mononuclear progenitor, suggests their common involvement in vascular protection and/or atherosclerotic plaque formation. Arrows indicate secreted proteins; dashed arrows indicate direct influences; in parenthesis are highlighted indirect relationships. EC = endothelial cells; SMC = smooth muscle cells; FB = fibroblasts; Mph = macrophages. (The hyperlink indicates the source of background drawing only).

These changes were meaningfully associated with cardiovascular pathology as expected. Pregnancy in itself seems to be the strongest amplifier of the OLRM in peripheral blood, in accord with a mobilization of cells of fetal origin. Encouragingly for a vascular determination of several neurodegenerative diseases, two conditions (MS and AD) also presented strong changes in the OLRM levels. MS presented a decrease in expression of the OLRM members, while AD presented an increase in expression of the OLRM members, corresponding to the known behavior of circulating progenitor cells in these pathologies.

Our findings warrant opening a line of experiments to directly confirm the origin of the OLRM in a given primitive cell class, and their functional relevance (as summarized in Diagram 3). If confirmed, these findings could establish a new collective biomarker for neurodegenerative conditions of vascular origin. Moreover, it is expected that targeting any of the OLRM members might modify coordinately all other members; this expands the power of drug searching significantly

As with other discoveries, the identification of OLRM raises as many new questions as problems it may solve. Among the most pressing are: a. *Cellular origin* of the detected genes; in fact, it is likely to be not a specific cell class, but the collective contributions of all PBMCs. *b. Control of covariation*; we expect this to be very complex, probably involving more than either one of the classical mechanisms (common promoters, co-localization on chromosomes, common miRNAs, epigenetic modifications etc.); c. *Functional meaning* of the membership: given that except OLR1 itself few other members are involved in lipid metabolism, we assume that in fact other function(s) of LOX-1 will eventually explain this gene cluster.

Acknowledgements

A special thanks to Dr. Leni Moldovan for her help with this project. The judges on the defense committee for this project included Dr. Derick H. Lindquist and Dr. Amanda A. Simcox. A great thanks to them for serving on the committee and also for providing me the knowledge from which stemmed my ability to do this project.

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